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ENVIRONMENTAL MICROBIOLOGY

Biofilm-Constructing Variants of *Paraburkholderia phytofirmans* PsJN Outcompete the Wild-Type Form in Free-Living and Static Conditions but Not *In Planta*

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ABSTRACT Members of the genus *Burkholderia* colonize diverse ecological niches. Among the plant-associated strains, *Paraburkholderia phytofirmans* PsJN is an endophyte with a broad host range. In a spatially structured environment (unshaken broth cultures), biofilm-constructing specialists of *P. phytofirmans* PsJN colonizing the air-liquid interface arose at high frequency. In addition to forming a robust biofilm *in vitro* and *in planta* on *Arabidopsis* roots, those mucoid phenotypic variants display a reduced swimming ability and modulate the expression of several microbe-associated molecular patterns (MAMPs), including exopolysaccharides (EPS), flagellin, and GroEL. Interestingly, the variants induce low *PR1* and *PDF1.2* expression compared to that of the parental strain, suggesting a possible evasion of plant host immunity. We further demonstrated that switching from the planktonic to the sessile form did not involve quorum-sensing genes but arose from spontaneous mutations in two genes belonging to an iron-sulfur cluster: *hscA* (encoding a cochaperone protein) and *iscS* (encoding a cysteine desulfurase). A mutational approach validated the implication of these two genes in the appearance of variants. We showed for the first time that in a heterogeneous environment, *P. phytofirmans* strain PsJN is able to rapidly diversify and coexpress a variant that outcompete the wild-type form in free-living and static conditions but not *in planta*.

IMPORTANCE *Paraburkholderia phytofirmans* strain PsJN is a well-studied plant-associated bacterium known to induce resistance against biotic and abiotic stresses. In this work, we described the spontaneous appearance of mucoid variants in PsJN from static cultures. We showed that the conversion from the wild-type (WT) form to variants (V) correlates with an overproduction of EPS, an enhanced ability to form biofilm *in vitro* and *in planta*, and a reduced swimming motility. Our results revealed also that these phenotypes are in part associated with spontaneous mutations in an iron-sulfur cluster. Overall, the data provided here allow a better understanding of the adaptive mechanisms likely developed by *P. phytofirmans* PsJN in a heterogeneous environment.

KEYWORDS *Paraburkholderia phytofirmans*, biofilm, competition, iron-sulfur cluster, plant defense, static cultures

The genus *Burkholderia* comprises members that occupy a wide variety of habitats, such as soil, rhizosphere, and water as well as humans and plants (1–3). The rhizosphere is the most common niche for beneficial plant-associated *Burkholderia*

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strains. It has been speculated that such preference may be associated with their catabolic versatility, which enables them to degrade root compounds and thus to persist on the root surface (4). Among the plant-associated *Burkholderia* strains, *Paraburkholderia phytofirmans* PsJN, recently classified in the *Paraburkholderia* clade (5), is a prominent and efficient plant growth-promoting endophyte (6–11). This strain, isolated from *Glomus vesiculiferum*-infected onion roots (8, 12), demonstrates a high adaptive capacity by colonizing the rhizosphere and internal tissues of a broad range of plant hosts (13). Interestingly, plant colonization by *P. phytofirmans* PsJN not only confers resistance against biotic factors (6, 14–16) but also protects the host against abiotic stresses (17–20).

The ability of *P. phytofirmans* strain PsJN to successfully colonize a wide variety of plant species might be based on its large genome (8.2 Mbp) encoding a broad range of physiological functions (13). Another hypothesis to explain its broad spectrum may be its ability to undergo phase variation or phenotypic switching. Indeed, phenotypic variation has an important adaptive role in the capacity of bacteria to colonize different host anatomical niches and to escape host defense mechanisms (21–23). This mechanism, often observed in *Burkholderia* spp., is a phenomenon that generates bacterial diversity and confers survival fitness in different environments (24–29). This process, by which bacteria undergo frequent and often reversible phenotypic changes, results from genetic or epigenetic alterations in specific loci of their genomes (21, 30, 31). It occurs at a high frequency of more than 10^{-5} switches per cell per generation (32) and can result in reversible ON or OFF expression of traits or in the variation of surface phenotypes. Several reports show that phenotypic variation is often involved in the production of exoenzymes and secondary metabolites and affects the colonization behavior and biocontrol activity of rhizosphere bacteria (33–36). It has an impact on different bacterial traits, such as cell surface components, adherence, exopolysaccharide (EPS) and lipopolysaccharide (LPS) production, and biocontrol activity (31, 37). This mechanism, associated with niche specialization and persistence in hosts, is well studied in opportunistic pathogen strains of *Burkholderia* and *Pseudomonas* spp. that infect hosts such as cystic fibrosis (CF) patients (38, 39) and in beneficial *Pseudomonas* spp. (36, 40), but to our knowledge, no information is available on plant-associated *Burkholderia* and *Paraburkholderia* strains.

In this study, we describe the spontaneous appearance of mucoid variants in *P. phytofirmans* strain PsJN from static broth cultures. Since colony morphology variation is known to modify host-bacterium interaction, we characterized these variants and showed that they are defective in flagellum-dependent motility and have enhanced surface adherence. These variants also showed an enhanced capacity to form biofilm *in vitro* and on *Arabidopsis* root surfaces. Interestingly, in contrast to the case for the wild type (WT), the PsJN biofilm-constructing specialists cannot induce *PR1* and *PDF1.2* gene expression in *Arabidopsis thaliana*. We further demonstrate that switching to the variant form did not involve quorum-sensing genes but arose from spontaneous mutations in two genes belonging to an iron-sulfur cluster (ISC) in *P. phytofirmans* PsJN.

RESULTS

***P. phytofirmans* PsJN mucoid variants emerge in spatially structured environments.** The *P. phytofirmans* PsJN::green fluorescent protein (GFP) WT strain usually produces a flat beige colony. When this strain was cultivated in static cultures, a pellicle at the air-liquid interface was observed. After plating, an alternate phenotypic form growing as convex and mucoid white colonies (Fig. 1) emerged spontaneously at a frequency of 5.10^{-4} per cell per generation. Under these conditions, the variant form was stable, as no reversion to the WT phenotype occurred. In shaken (180 rpm) cultures, the appearance of the variant form from the ancestral one was never observed. The growth kinetics of the initial WT strain and four variants isolated from four independent static cultures (V1 to V4) were compared in liquid shaken cultures and revealed to be identical (see Fig. S1 in the supplemental material). Here, we provide the first evidence

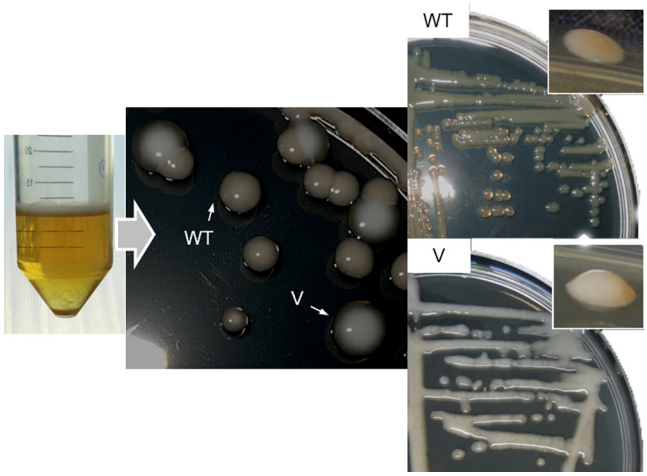

FIG 1 Emergence of *P. phytofirmans* PsJN variants from unshaken broth cultures. A single ancestral "WT" cell was propagated in 10 ml of King B medium at 28°C in a 50-ml microcosm without shaking for 8 days to produce a spatially heterogeneous environment. After 8 days, the culture was plated and showed substantial phenotypic diversity: a typical flat beige wild-type (WT) colony and an alternate phenotype, a mucoid white colony called variant (V).

that populations of *P. phytofirmans* rapidly diversify when propagated in unshaken broth cultures.

Variants form robust biofilm both on an abiotic surface and *in planta*. Certain bacterial traits can have an effect on niche construction, such as, for example, biofilm production that can modify the spatial structure and chemical environment (41–43). As surface attachment is the first step in biofilm formation, WT *P. phytofirmans* PsJN and the four variants were tested for adherence on abiotic surfaces. This adhesion assay revealed that the variants were 8- to 25-fold more adherent than the WT strain (data not shown).

To assess the ability of WT *P. phytofirmans* PsJN and variants to form biofilm, an *in vitro* crystal violet staining method was used. The quantitation of the biofilm biomass demonstrated that variants produced approximately 4-fold more biofilm than the WT strain ($P = 0.0001$) (Fig. 2A). We also studied *Arabidopsis thaliana* root attachment by WT *P. phytofirmans* PsJN or variants. To that end, *Arabidopsis* roots were observed by confocal scanning laser microscopy (CSLM) 4 days after inoculation (dai). The results indicated that *A. thaliana* roots were surrounded by a robust biofilm when inoculated with the variant cells, whereas the WT cells formed only microcolonies (Fig. 2B).

MAMPs are expressed differentially in the variants and the WT. EPS production has been reported to be involved in plant-bacterium interaction (44), and the capacity of variant cells to form a solid biofilm *in planta* may be correlated to EPS overproduction. The colony morphology observed on yeast extract-mannitol (YEM) plates suggested that all variants produced significantly more EPS than the WT (Fig. 3A). As motility is a bacterial trait often affected by colony morphology variation, the motility behavior of the WT and four variants was investigated. As shown in Fig. 3B, swimming motility, depending on flagella (45), was reduced in variant cells compared to WT cells after 30 h of incubation. To test whether the impaired motility of variants was due to a difference in flagellin production, surface proteins were extracted and separated by gel electrophoresis. A major band, with an estimated molecular weight of 40 kDa, appeared to be absent in V1, V2, and V3 and less intense in V4 than in the WT (see Fig. S2 in the supplemental material). This protein was identified by mass spectrometry as flagellin. Moreover, a second band (60 kDa) overexpressed in V1, V2, and V3 variant cells was identified as the GroEL chaperonin. To complete these data, observation by transmission electron microscopy (TEM) indicated that the variants had no flagellum or a truncated one compared to that of the WT (Fig. 3C). Thus, variants modulate the

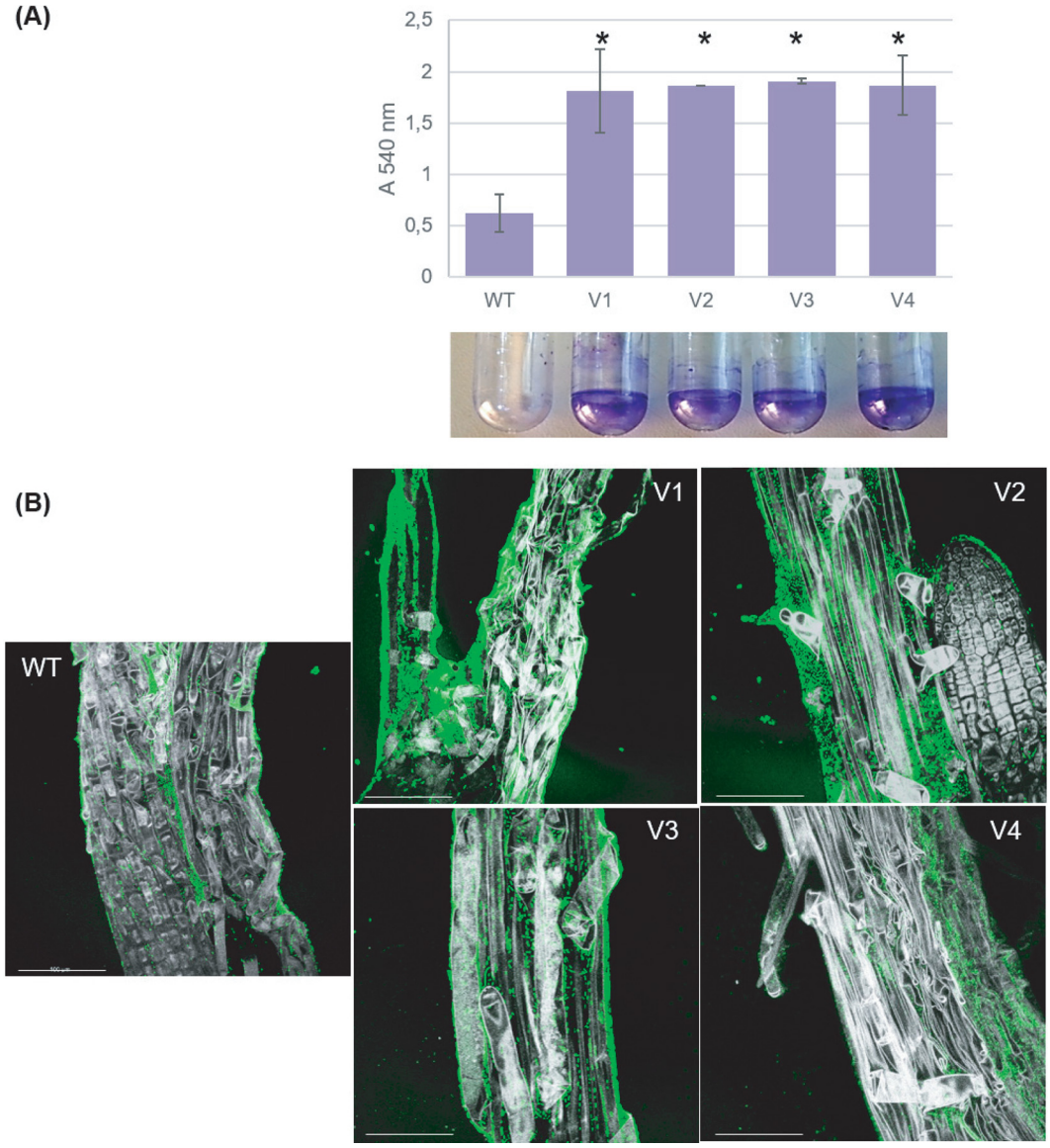

FIG 2 Biofilm formation on an abiotic surface and *in planta*. (A) Biofilms were quantified by crystal violet staining after 24 h of static incubation at 28°C. A graphical representation of the optical density readings (OD_{540}) derived from methanol elution of the crystal violet stain is shown. A partial *F* test was performed to determine if there were strain-specific differences in the level of biofilm formation (*, $P < 0.05$). (B) *A. thaliana* root attachment by *P. phytofirmans* PsJN::GFP 4 days after inoculation. Scanning confocal laser microscopy allowed visualization of green fluorescence emitted by the bacteria. Bars, = 100 μm.

expression of several microbe-associated molecular pattern (MAMPs) (flagellin, EPS, and GroEL).

PsJN biofilm-constructing specialists fail to induce common plant defense markers. Biofilm-constructing variants have an altered expression of several MAMPs and display an enhanced root adhesion, suggesting that they may be perceived differently from the WT by the host plant. To explore this hypothesis, 10-day-old *A. thaliana* plants were examined for reactive oxygen species (ROS) production (an early plant defense mechanism), expression of *PR1* (a well-known salicylic acid [SA]-dependent defense gene marker [46, 47]), and *PDF1.2* (a jasmonic acid/ethylene [JA/ET] plant defensin [48, 49]). In the first 2 h following bacterization, no ROS production was observed in response to either the WT or variants, whereas an oxidative burst was observed after challenge with the positive control, *Pseudomonas syringae* (50) (see Fig. S3 in the supplemental material). Inoculation with WT *P. phytofirmans* led to a signif-

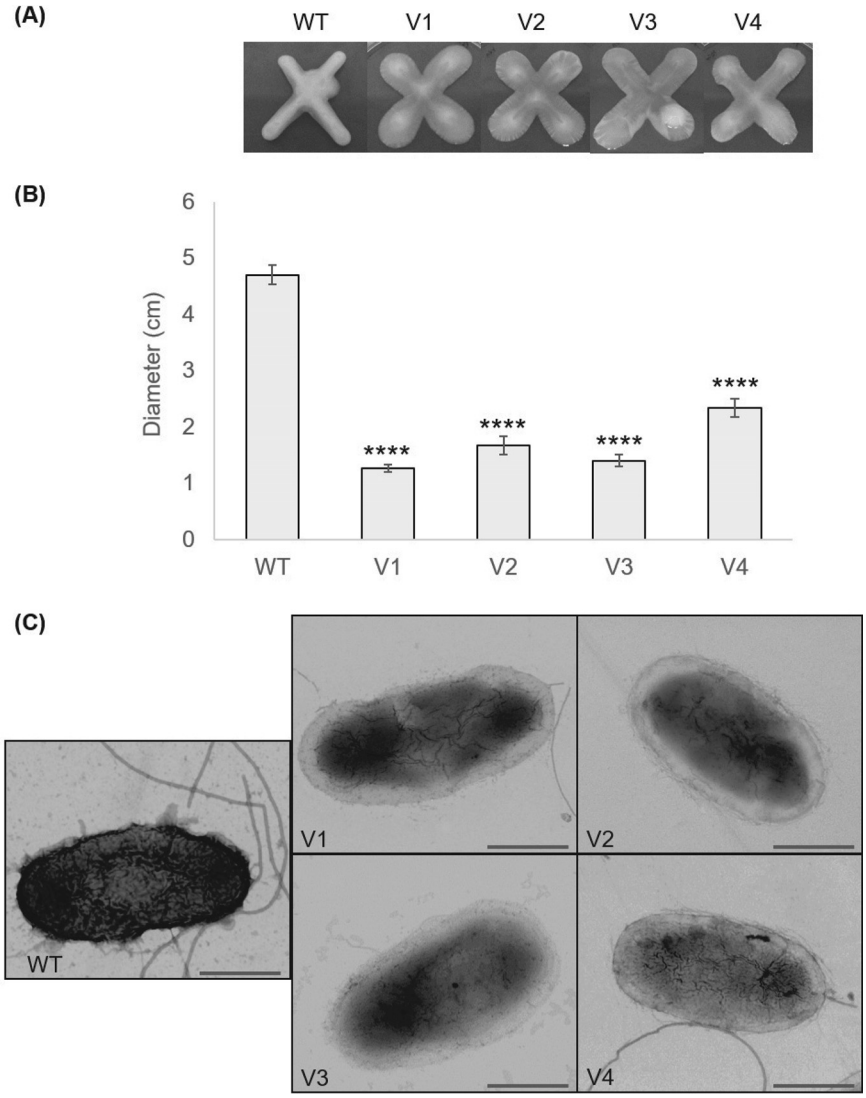

FIG 3 EPS production and motility in the wild type and variants. (A) EPS production by wild-type *P. phytofirmans* (WT) and variants (V1 to V4). EPS production was tested on mannitol medium containing 0.02% yeast extract. (B) Swimming motility (on LB plates with 0.2% agar without salt and supplemented with 0.1% Casamino Acids) was measured after 30 h of incubation. Error bars indicate standard errors (SE). Asterisks indicate a significant difference between the WT and others strains (****, $P < 0.0001$). (C) Negative-stain transmission electron micrographs (TEM) of WT *P. phytofirmans* PsJN and variants (V1 to V4). The strains were grown to mid-log phase in KB medium, transferred onto Formvar-coated nickel grids, and stained with phosphotungstic acid. Bars, = 0.5 μm.

icant induction of *PR1* and *PDF1.2* at 24 h after bacterial challenge (Fig. 4). Interestingly, variant forms led to an expression level comparable to those observed in response to *Escherichia coli*, used as a negative control, indicating a general effect of bacterization rather than a real recognition. Thus, we provide the first evidence that *P. phytofirmans* PsJN can generate a biofilm specialist subpopulation that are able to mask their MAMPs in order to reduce plant immune defenses.

Biofilm-constructing variants conserve PGP traits. Since *P. phytofirmans* PsJN has been described has having a plant growth-promoting (PGP) effect on various plants (13), PGP traits and the PGP effect on *A. thaliana* of different variants were compared to those of the WT. A positive effect of WT PsJN and all variants on root hair length was shown (see Fig. S4 in the supplemental material). Interestingly, in addition to their increased adhesion conferred by EPS overproduction, the variant forms maintain their positive effect on plant root development. No significant difference between variants

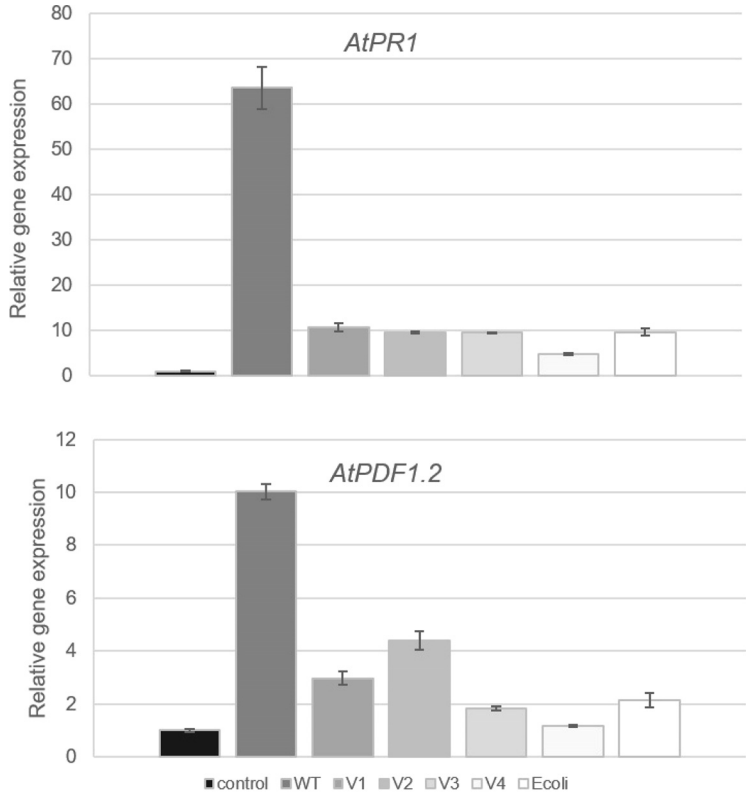

FIG 4 Defense gene expression in *A. thaliana* inoculated or not with *P. phytofirmans* PsJN (WT or V) or *E. coli*. Transcript accumulation of the *PR1* or *PDF1.2* gene was determined by quantitative real-time PCR at 24 h postinoculation with bacteria. Gene transcript levels were normalized using two reference genes (*UBQ5* and *EF1a*) as internal controls. Results are expressed as the fold increase in transcript level compared to that in control plants. Values shown are means ± standard deviations (SD) from two independent repetitions (each repetition was done in duplicates).

and the WT was observed in term of PGP traits, such as siderophore production, indole-3-acetic acid (IAA), phosphate solubilization (see Fig. S8 in the supplemental material), or carbon utilization (see Table S1 in the supplemental material).

Is the appearance of variants linked to an alteration of QS? Motility, mucoidy, and biofilm formation are under quorum-sensing (QS) control in *Burkholderia* spp. (51, 52). As these QS-regulated phenotypes are modified in the variants, we hypothesized that QS might be affected in the variants. *P. phytofirmans* PsJN possesses two *luxI-luxR* operons (Bphyt_0126/0127 and Bphyt_4275/4277), located on chromosomes 1 and 2, respectively, and one orphan *luxR* (Bphyt_6042), located on chromosome 2 (8, 13). The expression of the five corresponding genes was monitored by real-time PCR, and no significant difference was observed between WT and variant cells (see Fig. S5A in the supplemental material). Moreover, no significant difference was observed between WT and variant cells in term of *N*-acyl homoserine lactone (AHL) production (Fig. S5B). Our data allow the conclusion that the phenotypic differences observed between the planktonic and the biofilm forms cannot be attributed to a differential expression of QS genes or molecules.

The appearance of variants is correlated with mutations in *hscA* and *iscS*, genes belonging to an Fe-S cluster. To determine whether these different phenotypes were due to a genetic alteration, whole-genome sequencing of the four colonial variants (V1 to V4) and the parental WT strain was carried out using Illumina technology. We selected variations (single nucleotide polymorphisms [SNPs] and indels) in V1, V2, V3, and V4 compared to their ancestor WT strain. The identified mutations targeted two genes, *iscS* and *hscA*, encoding a cysteine desulfurase and a cochaperone, respectively (Table 1). They both belong to an iron-sulfur cluster (ISC) system, encoded by the

TABLE 1 List of genetic variations in *P. phytofirmans* PsJN variants[a]

Variant	Locus	Gene	Gene product	Position	WT base	Variant base	Type	Coding region change	Position (amino acids)	Amino acid change
V1	BPHYT_RS12760	*hscA*	Chaperone protein HscA	2909360	CGCG		4-bp deletion	WP_012433563.1	535	Ala → fs
V2	BPHYT_RS12780	*iscS*	Cysteine desulfurase IscS	2912793	A	G	SNV	WP_012433567.1	390	Trp → Arg
V3	BPHYT_RS12780	*iscS*	Cysteine desulfurase IscS	2912793	A	G	SNV	WP_012433567.1	390	Trp → Arg
V4	BPHYT_RS12780	*iscS*	Cysteine desulfurase IscS	2913221	A	C	SNV	WP_012433567.1	247	Leu → Arg

[a]SNV, single nucleotide variant; fs, frameshift.

iscSUA-hscBA-fdx-iscX cluster (Fig. 5). In order to validate that the appearance of variants is correlated to mutations in *hscA* or *iscS*, we constructed deletion mutants affected in each gene (for the methodology, see Fig. S6 in the supplemental material). The results showed that mutants had a reduced motility (Fig. 6A) identical to the one observed for variant cells and an enhanced ability to produce EPS (Fig. 6B) and to form biofilm *in vitro* (Fig. 6C). Moreover, *A. thaliana* gene expression analysis showed that, as for the variants, inoculation with the Δ*hscA* or Δ*iscS* mutant induces a lower expression of *PDF1.2* and *PR1* than inoculation with the WT (Fig. 6D).

Variants outcompete wild-type cells in free-living and static conditions but not *in planta*. In order to determine if the WT and variant populations may differ in term of competitiveness, we performed bacterial competition assays under free-living (on a plate), shaking, and static conditions. For this purpose, two strains were mixed in equal numbers and incubated in shaking or static cultures for 24 h or on agar plates for 72 h, and each subpopulation was enumerated using a fluorescent tag. In shaking condition, the sizes of the WT and V populations did not differ significantly. On the other hand, plate and static competition assays between the WT (dsRed) and V::GFP forms revealed that all V forms outcompeted the WT (Fig. 7A). However, when the same competition assays were performed between the WT::GFP strain and each of the two mutants (Δ*hscA* and Δ*iscS* mutants) tagged with dsRed, the results indicated that the two populations did not differ significantly on plates but clearly demonstrated that in shaking and static conditions, the WT form outcompeted the mutants (Fig. 7B).

When competition assays were performed *in planta,* the WT form clearly outcompeted both the V forms (Fig. 8A) and mutants (Fig. 8B) during all steps of colonization (2, 6, and 12 dai).

DISCUSSION

In this study, we showed for the first time that *P. phytofirmans* PsJN, a well-studied endophyte PGP rhizobacterium (PGPR), is able to generate biofilm-constructing variants from static liquid microcosms. Variants have been isolated from a single ancestral *P. phytofirmans* PsJN "WT" cell propagated for 8 days without shaking to produce a spatially heterogeneous environment (41). Previous studies reported adaptive diversification and niche partitioning in bacterial populations cultivated in such experimental structured environments (53, 54). Phenotypic diversity is typical of bacteria that occupy heterogeneous ecological niches and has been related to adaptation to different environmental situations and to sudden changes in the ecosystem. Under these

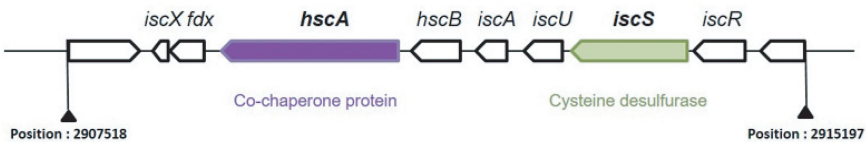

FIG 5 Genetic organization of the *P. phytofirmans* PsJN Iron Sulfur Cluster (ISC) system. *iscR* transcriptional regulator, Rrf2 family; iron-sulfur cluster assembly transcription factor IscR (WP_012433568.1); *iscS* cysteine desulfurase (WP_012433567.1); *iscU* FeS cluster assembly scaffold IscU (WP_012433566.1); *iscA* iron-sulfur cluster assembly protein IscA (WP_012433565.1); *hscB* chaperone protein HscB (WP_012433564.1); *hscA* cochaperone protein HscA (WP_012433563.1); *fdx* ferredoxin 2Fe-2S type (WP_012433562.1); *iscX* FeS assembly protein IscX (WP_007181290.1).

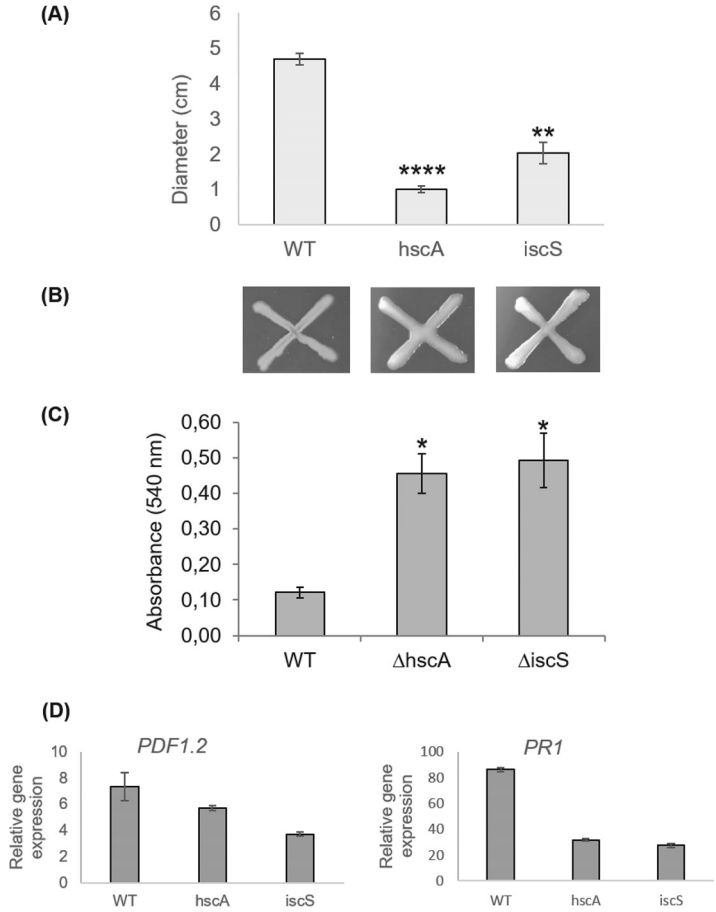

FIG 6 Characterization of Δ*hscA* and Δ*iscS* mutants. (A to C) Motility (A), EPS production (B), and biofilm (C) of Δ*hscA* and Δ*iscS* mutants compared to WT cells. (D) Defense gene expression in *A. thaliana* inoculated or not with *P. phytofirmans* PsJN (WT or mutants) or *E. coli*.

conditions, phase variation would generate a mixed population able to colonize different parts of the ecosystem, and in the case of rapid environmental changes, part of the initial population would survive (30). Though this phenomenon was described mainly for bacteria belonging to *Pseudomonas* spp., it was also often observed in *Burkholderia* spp. (24–29).

Here, we provide for the first time evidence that a bacterium belonging to the *Paraburkholderia* clade is able to perform phenotypic switching. The characterization of variants revealed that mucoid variants of PsJN showed a reduced motility related to the lack of a flagellum, an overproduction of EPS, and an enhanced ability to form biofilm *in vitro*. This better ability to form biofilm may confer to these variants significant fitness advantages, since colonizing a new niche usually required growth at a surface and formation of a biofilm (55). EPS production has also been reported to be involved in plant-bacterium interaction (44), and the capacity of variant cells to form a solid biofilm *in planta* may be correlated to EPS overproduction. Interestingly, we showed that the PsJN biofilm specialists have an enhanced ability to adhere on *Arabidopsis* root surfaces. This property may give them a strong selective advantage, since successful plant colonization and bacterial persistence rely on the bacterium's ability to form adherent microbial populations (56, 57). A better initial attachment to *Medicago* roots was directly related to a higher EPS production in *Rhizobium leguminosarum* (58), and in *Bacillus subtilis*, EPS is required to form robust biofilm *in vitro* and *in planta* (59, 60). However, even if variants overproduced EPS, our data on bacterial competition indicated that the variant form outcompeted the WT in free-living conditions but not *in planta*. It would be relevant to study the impact of EPS overproduction on soil

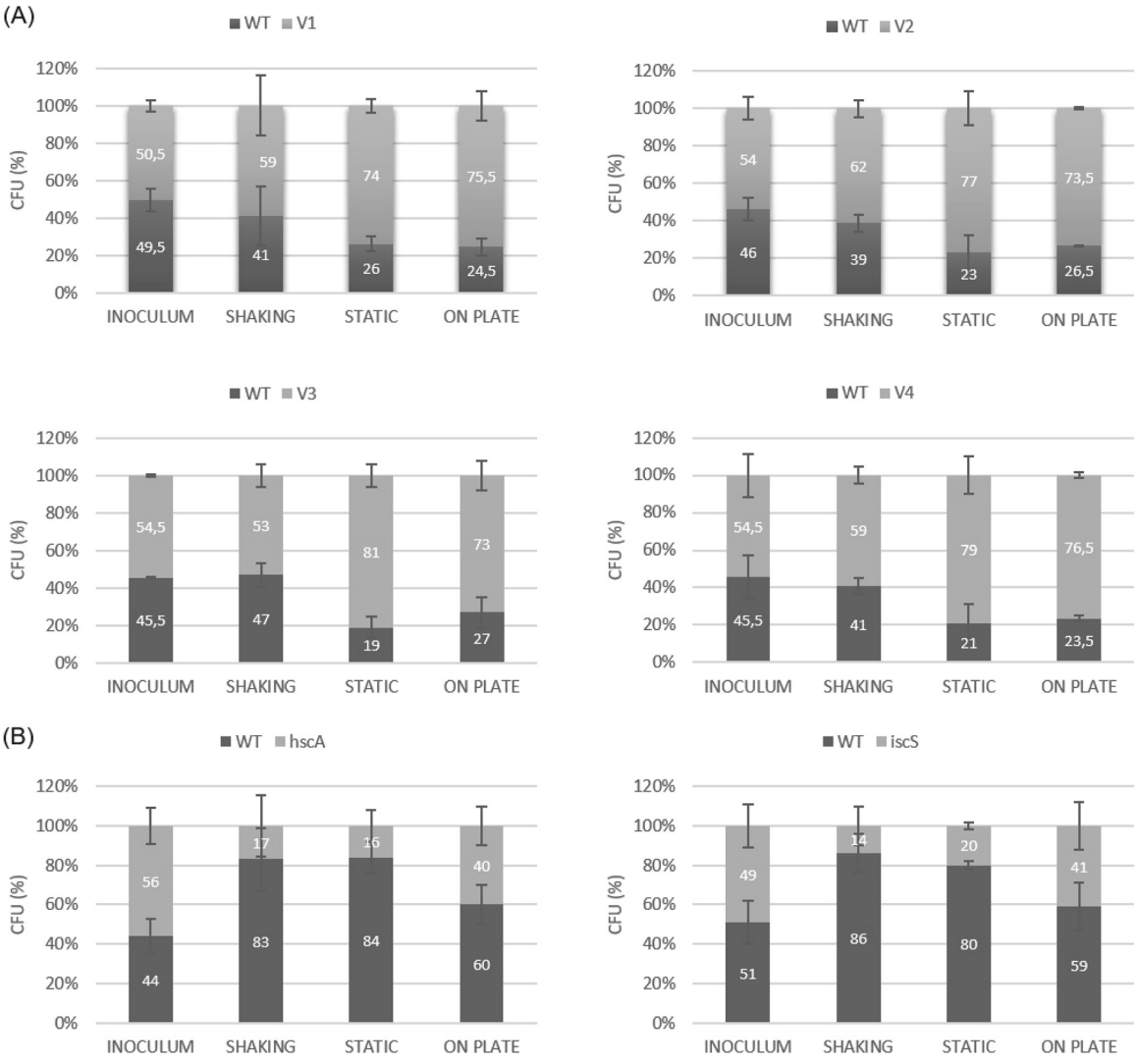

FIG 7 *In vitro* bacterial competition. A mixture of dsRed-tagged WT *P. phytofirmans* and GFP-tagged *P. phytofirmans* V (A) or of GFP-tagged WT *P. phytofirmans* and dsRed-tagged *P. phytofirmans* mutant (B) was tested for competition after 72 h on plates and 24 h in shaking and static cultures. The percentage of CFU of the two competing strains in the inoculum and recovered after 72 h (on plate) or 24 h (shaking and static) of contact is shown. Error bars indicate standard deviations (SD). At least 3 independent replicates were analyzed.

properties and on protection of plants against abiotic and biotic stresses. Indeed, EPS production by PGPRs may have an impact on soil aggregation (61, 62) and may help plants to survive under water-limiting conditions (63).

Our results underlined that wild-type PsJN and mucoid variants differ in their expression of MAMPs (flagellin and EPS), suggesting that the perception of *P. phytofirmans* PsJN by host plants may be different in response to the wild type or variants. Indeed, MAMPs are recognized by the plant innate immune system *via* pattern recognition receptors (PRRs) present at the plant cell surface. This recognition leads to a basal resistance called MAMP-triggered immunity (MTI) (64). EPS overproduction has an impact on host immunity, since by masking bacterial surface structures no longer recognized by neutrophils and macrophages, it can facilitate bacterial persistence in a mouse model of infection (65). In plants, EPS is also known to suppress MTI (66). Flagellin, the main component of flagella, is a well characterized MAMP recognized by the plant receptor FLS2 (67). The flg22 peptide was previously described to be an active MAMP of *P. phytofirmans* PsJN inducing MTI responses in grapevine and *Arabidopsis* (68), and a PsJN mutant deficient in flagella (Δ*fliA*) did not induce *PR1* gene expression (16). PsJN variants also overproduced GroEL. GroEL was recently described as a

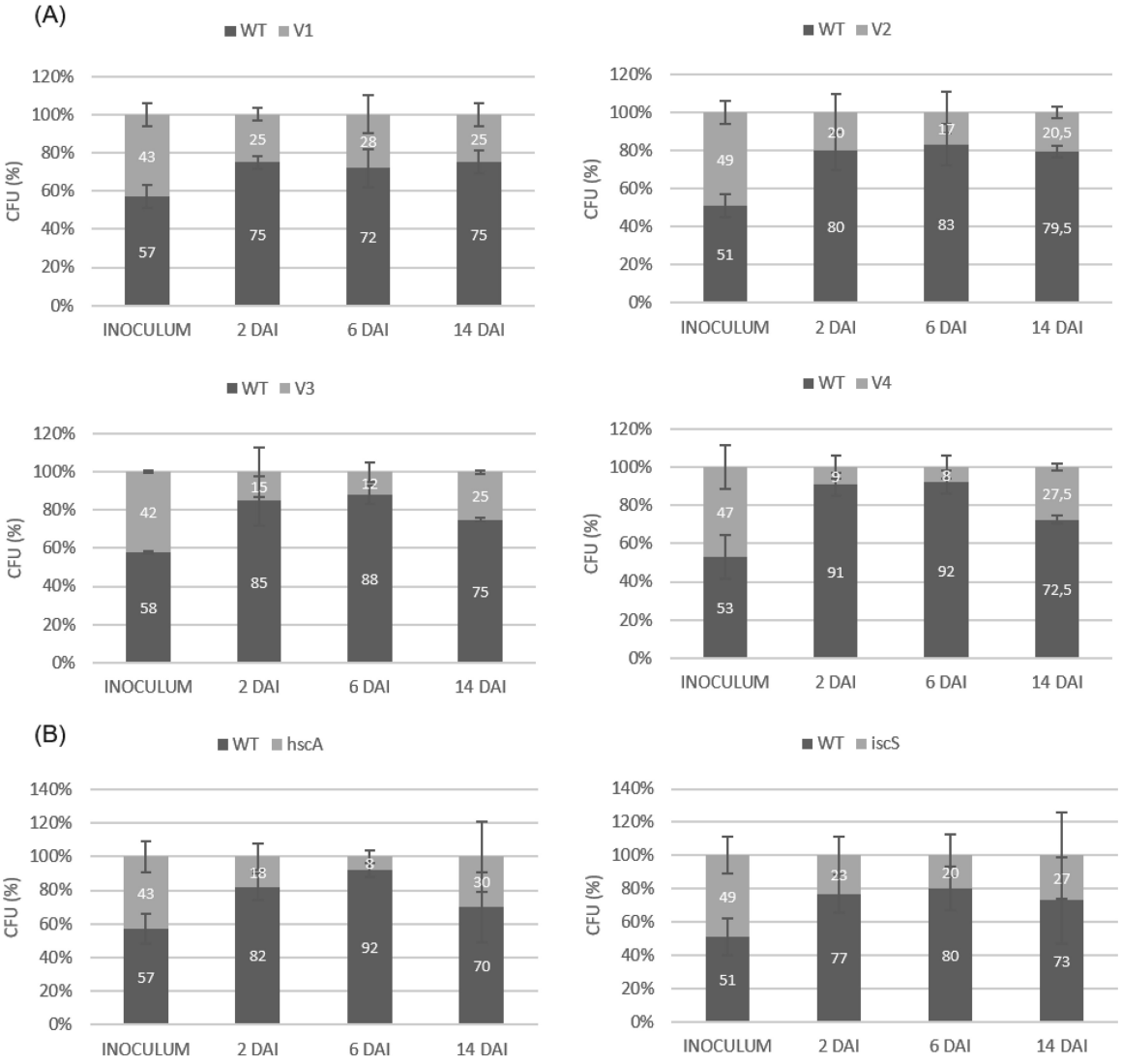

FIG 8 *In planta* bacterial competition. A mixture of dsRed-tagged WT *P. phytofirmans* and GFP-tagged *P. phytofirmans* V (A) or of GFP-tagged WT *P. phytofirmans* and *P. phytofirmans* mutant dsRed-tagged (B) was tested for *in planta* competition at 2, 6, and 12 days after inoculation (dai). The percentage of CFU of the two competing strains in the inoculum and recovered at 2, 6, and 12 dai (*in planta*) is shown. Error bars indicate standard deviations (SD). At least 2 independent repetitions (each in duplicates) were analyzed.

pathogen-associated molecular pattern (PAMP) that triggers PAMP-triggered immunity (PTI) in *Arabidopsis* (69). Finally, GroEL plays a significant role in the adherence and subsequent biofilm formation in many bacteria (70). PGPRs were previously described to use phase variation to avoid stimulation of the host's immune system (23, 36). However, in the case of PsJN, this property does not allow the variants to be more competitive *in planta*, indicating that other properties relevant for maintenance in plants may have been lost by the variants.

Whole-genome sequencing of the four colonial variants (V1 to V4) and the parental WT strain brought to light that appearance of variants was correlated with spontaneous mutations occurring in *hscA* or *iscS*, encoding, respectively, a cochaperone protein and a cysteine desulfurase belonging to an iron-sulfur cluster (ISC) system. In *E. coli*, the ISC system is thought to serve as a housekeeping assembly system under normal conditions (71) and to participate into the maturation of a large variety of Fe/S proteins. Our mutational approach confirmed that these genes were implicated in some traits observed, as mutants have enhanced abilities to produce EPS and to form biofilm *in vitro* and a decrease in motility. These results are in accordance with those observed in

E. coli (72). Indeed, a link between Fe/S homeostasis and cell surface properties was previously established, with different Fe/S biogenesis mutants having modified swarming motility (73) or modified cell surface properties such as mucoidy, motility, and biofilm formation (74). The iron-sulfur (Fe-S) cluster is a protein cofactor that is essential for various biological activities. However, in our *in vitro* competition assays (on plates and in shaking and static conditions), mutants behaved differently from variants. These results indicated that other mechanisms may be involved in the switch from planktonic cells to biofilm variants in PsJN, such as differential regulation of a global regulator or epigenetic mechanism (DNA methylation).

In conclusion, we highlighted the ability of PsJN to generate subpopulations that differ in motility, biofilm ability, EPS production, root adhesion, and plant defense responses. Since heterogeneity of populations in bacteria can be considered a strategy helpful for adaptation to adverse conditions, it would be interesting to study different stress conditions in which the variant has a competitive advantage over the wild type and to tentatively link this heterogeneity to the ability of this PGPR endophyte to colonize a broad host spectrum.

MATERIALS AND METHODS

Strains and growth conditions. The strains, plasmids, and primers used in this study are listed in Table 2. *Escherichia coli* WM3064 was grown in LB containing diaminopimelic acid (DAP) at 100 mg/liter. *Saccharomyces cerevisiae* InvSc1 was grown on yeast extract-peptone-dextrose medium at 30°C (75). *Paraburkholderia phytofirmans* strain PsJN::GFP and PsJN expressing dsRed were cultivated in King B (KB) liquid medium (76) supplemented with kanamycin (Km) at 50 μg/ml ($KBKm_{50}$) or chloramphenicol (Cm) at 25 μg/ml ($KBCm_{25}$), respectively, at 28°C on a shaker (180 rpm) overnight. Bacterial cells were then collected by centrifugation at 4,500 $\times$ *g* for 15 min at 4°C and resuspended in phosphate-buffered saline (PBS) (containing, per liter, 10 mM NaH_2PO_4 with 0.8% NaCl, pH 6.8), after which the optical density at 600 nm (OD_{600}) was adjusted to 10^9 CFU/ml with PBS (OD = 0.8). When required, Km (50 μg/ml), Cm (25 μg/ml), and gentamicin sulfate (Gm) (25 μg/ml for *E. coli* and 50 μg/ml for *P. phytofirmans*) were added.

Isolation of variants from static microcosms. Populations were isolated from a single ancestral "WT" cell, propagated in 10 ml of KB with Km, and incubated at 28°C in a 50-ml microcosm. Microcosms were incubated without shaking to produce a spatially heterogeneous environment (41). After 8 days, populations show phenotypic diversity, which is observed after plating. The fact that variants carried the GFP marker and sequencing of the 16S rRNA gene confirmed that the variants were not contaminants.

Detection of AHLs. For quantification of *N*-acyl homoserine lactones (AHLs), cultures were grown in KB medium for 20 h. AHLs extracted from cell-free culture supernatants (equivalent to 1 ml of supernatant) were separated by thin-layer chromatography (TLC) and detected using an overlay of agar seeded with *Agrobacterium tumefaciens* NTL1(pZLR4), as described previously (77). The following standards were used: oxo-substituted standards 3*O*,C_6-homoserine lactone (3*O*,C_6-HSL), 3*O*,C_8-HSL, and 3*O*,C_{10}-HSL and unsubstituted standards C_6-HSL, C_8-HSL, C_{10}-HSL, and C_{12}-HSL.

Whole-genome sequencing and SNP/indel identification. Paired-end libraries with an insert size of 270 to 390 bp were constructed for the ancestral strain (WT) and its variants V1, V2, V3, and V4. DNA sequencing was performed with Illumina HiSeq 2000 v3 technology using the 2 $\times$ 150-bp paired-end read module at the CNRS IMAGIF platform (Gif-sur-Yvette). After quality (quality score threshold, 0.05) and length (above 40 nucleotides) trimming, paired-end reads were mapped against the published sequence of strain PsJN (GCA_000020125.1) (78) at a mild stringency threshold (0.8 identity on 0.5 read length) using CLC Genomics Workbench version 7.0.0 software (CLC Inc, Aarhus, Denmark). After mapping, mean genome coverage was over 400-fold. The variant calling tool from CLC genomic workbench version 7.0.0 was used for detection of variations (SNPs and indels) in V1, V2, V3, and V4 compared to their ancestor WT strain.

Adherence assays. Abiotic adherence assays were performed. Briefly, bacteria were grown in $KBKm_{50}$ medium at 28°C on a shaker (180 rpm) for 20 h, washed twice, and resuspended in PBS. Approximately 2 $\times$ 10^8 CFU was added in duplicate to the wells of a six-well polystyrene plate (BD Biosciences, Mississauga, ON, Canada) containing 2 ml of PBS. Additionally, serial 10-fold dilutions were performed on the initial inoculum, and 100 μl of each dilution was plated on KB agar plates to determine precisely the number of bacteria added to each well (input). The six-well plates were centrifuged for 2 min at 300 $\times$ *g* and incubated at 28°C for 4 h. Each well was washed five times with 2 ml of PBS, and bound bacteria were detached from the surface by the addition of 500 μl of 10 mM EDTA and 1.5 ml of 1% (vol/vol) Triton X-100. Bacteria were then centrifuged at 4,500 $\times$ *g* for 4 min, and the pellet was resuspended in 1 ml PBS. Serial 10-fold dilutions were prepared, and 100 μl of each dilution was plated on KB agar plates and incubated at 28°C. Colony enumeration was performed to determine the number of bacteria that remained adherent to the six-well plates after the PBS washes (output). The adherence of each strain tested was calculated by dividing the output number by the input number and expressed relative to the adherence of the *P. phytofirmans* PsJN WT strain, which was set as 1. The experiment was repeated independently three times.

TABLE 2 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence	Reference or source
Strains		
Paraburkholderia phytofirmans		
PsJN	Wild type	Lab stock
PsJN::GFP	PsJN tagged with GFP; Km^r	Lab stock
PsJN Δ*hscA*	Mutant with deletion of *hscA* gene	This study
PsJN Δ*iscS*	Mutant with deletion of *iscS* gene	This study
PsJN::dsRed	PsJN tagged with dsRed; Cm^r	This study
Saccharomyces cerevisiae InvSc1	Yeast strain for *in vivo* recombination; *MAT***a**/*MATα leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52 his3-Δ1/his3-Δ1*	Invitrogen (Carlsbad, CA, USA)
Escherichia coli		
WM3064	Strain for conjugation; λpir, DAP auxotroph	85
DH5α	Host for cloning	Lab stock
Plasmids		
pin29	Nonmobilizable plasmid pIN29; ori_{pBBR} Δ*mob* Cm^r dsRed	83
pMQ30	7.6-kb mobilizable suicide vector used for gene replacement in *Pseudomonas*: *sacB URA3* Gm^r	75
pMQ30-ΔhscA	pMQ30 containing two fragments of ISC genes Bphyt_2574 and Bphyt_2576	This study
pMQ30-ΔiscS	pMQ30 containing two fragments of ISC genes Bphyt_2578 and Bphyt_2580	This study
Primers (5′ → 3′)		
UphscAF	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGGAACGACAGCCACTTCGAC	This study
UphscAR	GCTTCACCACCACAGATGCCTTGCCACGACCCATGAAG	This study
DhscAF	CTTCATGGGTCGTGGCAAGGCATCTGTGGTGGTGAAGC	This study
DhscAR	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATTTCGACAACCAGATCCTGCT	This study
UpiscSF	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGCGATGATTGACCTGGCACTG	This study
UpiscSR	TTTCCGTCGCCATTTCTTCGACCCGAGGTCCAGATGATTT	This study
DiscSF	AAATCATCTGGACCTCGGGTCGAAGAAATGGCGACGGAAA	This study
DiscSR	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATCCGTGACGTTGCTTGTAGTC	This study
Bphyt_0126F	CGTCATCCCCTTCGGTATGG	This study
Bphyt_0126R	ATACGGGAACAGGGACATGC	This study
Bphyt_0127F	GATCGTATGCCTGACCTGGG	This study
Bphyt_0127R	CCTGCATTGCCTTTCCGATG	This study
Bphyt_4275F	ATGCTGAAGGACACCTTCCC	This study
Bphyt_4275R	AAAGCCGAACGACTGTTCCT	This study
Bphyt_4277F	TGTTATTGCGCGACCTGGTA	This study
Bphyt_4277R	GGTCCATTTCAGGCATTCGC	This study
Bphyt_6042R	TTGCCTACTTTCTCGGGCTG	This study
Bphyt_6042F	CACATAGGCCTGAGCGACAT	This study
16S-F	AAGCCTGATCCAGCAATGCC	This study
16S-R	TCCGATTAACGCTTGCACCC	This study

Swimming motility, biofilm formation, and EPS production. Swimming motility was determined on plates of LB without salt supplemented with 0.1% Casamino Acids that were solidified with 0.2% agar and measured after 30 h of incubation as described previously (79). Overnight bacterial cultures (5 μl) were spotted in the middle of plates and then incubated at 28°C for 30 h.

Overnight cultures grown at 28°C for 16 h were diluted to an OD_{600} of 0.2 in KB, and triplicate 500-μl aliquots were dispensed into polystyrene tubes. Following 24 h of static incubation at 28°C, the medium was removed and the tubes were washed gently with distilled water. Adherent bacteria were stained with 1% (wt/vol) crystal violet and washed three times with distilled water. The bound crystal violet was dissolved in 1 ml of 100% methanol and quantified by measuring the absorbance at 540 nm. The experiment was repeated independently three times in triplicates.

Exopolysaccharide (EPS) production by WT PsJN and variant forms was tested on mannitol medium containing 1% mannitol and 0.06% yeast extract (79).

RNA extraction and quantitative real-time PCR. For *PR1* and *PDF1.2* gene expression in *Arabidopsis thaliana*, seeds were sterilized as described previously (68). Five or six seeds were then dispensed into each well of 12-well tissue culture plates with 2 ml of Murashige-Skoog (MS) medium with vitamins (Duchefa M0256) supplemented with 5 g/liter sucrose and 0.5 g/liter 2-(*N*-morpholino)ethanesulfonic acid, pH 5.8. Seedlings were grown at 22°C in a 12-h photoperiod at a light intensity of 100 $\mu m\ s^{-1}$ for 10 days before treatment. On the eighth day, the media were replaced with 2 ml of fresh media. Seedlings were elicited by WT *P. phytofirmans* and variants (V1 to V4) at a final concentration of 10^7 CFU/ml. Seedlings were incubated for 24 h at 22°C. The samples were then harvested, total RNA was isolated using Extract'All (Eurobio), and 1 μg of DNase-treated RNA was used for reverse transcription using the ABsolute Max 2-Step QRT-PCR SYBR green kit (ThermoElectron) according to the manufacturer's instructions. The transcript levels were determined by quantitative real-time PCR as previously described (80).

For QS gene expression in *P. phytofirmans* PsJN (WT and V), RNA was extracted from overnight (16-h) cultures using Extract'All (Eurobio), and 1 μg of DNase-treated RNA was used for reverse transcription using the ABsolute Max 2-Step QRT-PCR SYBR green kit (ThermoElectron) according to the manufacturer's instructions. The reference gene was the 16S rRNA gene. Gene expression differences between WT PsJN and variants were calculated using the $2^{-\Delta\Delta CT}$ formula. Primers are listed in Table 2.

Competition assays *in vitro* and *in planta*. Competition assays were conducted as described previously (79) with modifications. The GFP- and dsRed tagged-strains of *P. phytofirmans* PsJN were grown overnight in 5 ml KB medium supplemented with Km or Cm, respectively. After washing the cultures twice in KB, the OD_{600} of each culture was adjusted to 0.8 (10^9 CFU/ml), and the cultures were subsequently diluted 10,000-fold. These diluted suspensions were plated on KB with the appropriate antibiotic to enumerate precisely the number of initial CFU for each strain. For competition experiments on plates, diluted suspensions of bacteria of interest (WT-dsRed/V::GFP or WT::GFP/mutant-dsRed) were mixed 50:50 (vol/vol) and immediately diluted, and 5-μl drops were spotted onto a nonselective KB plate and incubated for 72 h at 28°C. Colonies of each subpopulation were enumerated on drops using the fluorescent tag.

For competition in shaking and static cultures, suspensions (adjusted to 10^9 CFU/ml) of the bacteria of interest (WT-dsRed/V::GFP or WT::GFP/mutant-dsRed) were mixed 50:50 (vol/vol) (final concentration, 10^7 CFU/ml) and cultivated at 28°C on a shaker (180 rpm) (shaking) or without shaking (static) overnight. Subsequently, diluted suspensions were plated on nonselective KB (5-μl drops) and incubated for 72 h at 28°C. Colonies of each subpopulation were enumerated on drops using the fluorescent tag.

For *in planta* competition, the bacterial mixture (WT-dsRed/V::GFP or WT::GFP/mutant-dsRed) containing around 10^2 CFU of each strain was immediately applied on *A. thaliana*. The plants were grown in a sterilized soil mix (vermiculite-perlite-soil; 1:1:1 [vol/vol/vol]) and regularly watered. Two independent experiments with 4 plants each were performed. At 2, 6, and 12 dai, roots were removed from soil and then ground with 1 ml of PBS. The homogenate was serially diluted in 10-fold steps and cultured on KB medium plates (triplicates) with the appropriate antibiotic. The bacterial colonies were counted after 3 days of incubation at 28°C.

CLSM and TEM. To investigate the putative presence of biofilms on root surface, a TCS SP2 (Leica, Manheim, Germany) confocal laser scanning microscopy (CLSM) system, equipped with a 40× oil immersion objective and with a numerical aperture of 1.4, was used. For the observations, we used several lasers for excitation: a 488-nm argon visible laser, a 351-nm laser, and a 364-nm UV laser. Prior to observations, the roots were harvested, washed with PBS, and then placed on a microscope slide with a cover glass before being positioned onto the objective. The reflected fluorescent light was selected through a pinhole of 1 Airy unit. Acousto-optical tunable filters (AOTF) selected light from 408 to 478 nm (autofluorescence from lignin; lookup table = red), from 493 to 537 nm (fluorescence of GFP; lookup table = green), and from 613 to 698 nm (autofluorescence; lookup table = red). Images were collected using the following parameters: 1× zoom factor, 512 by 512 pixels, and frequency of 400 Hz [81]). Confocal pictures were the averages of 10 acquisitions. Each experiment was repeated independently three times, and representative images from the overall observations are presented. For transmission electron microscopy (TEM) observations, early-exponential-phase bacteria were washed in PBS (pH 6.8). Formvar-carbon-coated copper grids (200 mesh) were floated onto a drop of washed bacteria, rinsed in ultrapure-grade water, and negatively stained with 0.5% (wt/vol) phosphotungstic acid (10 s). Electron microscopy was performed with a Jeol 1,200× transmission electron microscope.

Construction *of P. phytofirmans* PsJN Δ*hscA* and Δ*iscS* mutants. *P. phytofirmans* PsJN Δ*hscA* and Δ*iscS* mutants were constructed using *in vivo* homologous recombination in the yeast *S. cerevisiae* InvSc1, as previously described (82). The allelic replacement vector pMQ30 (75) was used for mutant construction, and upstream and downstream regions of the *hscA* and *iscS* genes were amplified using primers UphscAF and UphscAF (908-bp amplicon) and primers DhscAF and DhscAR (826-bp amplicon) for the *hscA* gene and primers UpiscSF and UpiscSF (800-bp amplicon) and primers DiscSF and DiscSR (884-bp amplicon) for the *iscS* gene, respectively (Table 2). The PCR products were cloned flanking each other through *in vivo* homologous recombination in the yeast *S. cerevisiae* InvSc1. Plasmids pMQ30-Δ*hscA* and pMQ30-Δ*iscS* were extracted from *S. cerevisiae* InvSc1 and then introduced into *E. coli* WM3064 by electroporation. The plasmids were mobilized into *P. phytofirmans* PsJN by conjugation (see Fig. S6 in the supplemental material), and deletion of the *hscA* and *iscS* genes was confirmed by PCR.

Fluorescent tagging of *Paraburkholderia phytofirmans*. dsRed fluorescent tagging of *P. phytofirmans* wild-type PsJN and the Δ*hscA* and Δ*iscS* mutants was carried out by electroporation using the plasmid pIN29 carrying the dsRed-encoding gene and a chloramphenicol resistance cassette (83). Transformants were selected on LB plates with chloramphenicol (25 μg/ml).

Protein extraction and analysis. Protein extraction was carried out as described previously (36) with some modifications. Bacteria were cultivated in KB medium overnight and washed twice with PBS.

ROS production. ROS production was measured as described previously (84). The final bacterial elicitation solution was adjusted to an OD_{600} of 0.1, corresponding to 10^8 CFU/ml).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02670-18.

SUPPLEMENTAL FILE 1, PDF file, 2.6 MB.

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We declare no conflict of interest.

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